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**Keywords:** Biomolecular NMR; Cardiovascular disease; Cardiovascular risk; Cholesterol efflux ; Coronary artery disease; High-density lipoprotein

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## Direct estimation of HDL-mediated cholesterol efflux capacity from serum

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*Running title:* Estimation of HDL-mediated cholesterol efflux.

*Keywords:* Cholesterol, Cholesterol efflux, High-density lipoprotein, Coronary artery disease, Cardiovascular disease, Biomolecular NMR.

*Abbreviations:* HDL, high-density lipoprotein; HDL-CEC, cholesterol efflux capacity of HDL; CHD, coronary heart disease; ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; SR-B1, scavenger receptor B1; cAMP, cyclic adenosine monophosphate; ApoA1, apolipoprotein A1; HDL-P, total HDL particle concentration; NMR, nuclear magnetic resonance spectroscopy; MI, myocardial infarction; CVD, cardiovascular disease; IDI, integrated discrimination improvement index; NRI, net reclassification index; RR, risk ratio; BODIPY; boron-dipyrromethene.

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1   **ABSTRACT**

2   **BACKGROUND:** High-density lipoprotein mediated cholesterol efflux capacity (HDL-CEC) is  
3   a functional attribute that may have a protective role in atherogenesis. However, the estimation of  
4   HDL-CEC is based on *in vitro* cell assays that are laborious and hamper large-scale phenotyping.

5   **METHODS:** Here, we present a cost-effective high-throughput nuclear magnetic resonance  
6   (NMR) spectroscopy method to estimate HDL-CEC directly from serum. We applied the new  
7   method in a population-based study of 7,603 individuals including 574 who developed incident  
8   coronary heart disease (CHD) during 15 years of follow-up, making this the largest quantitative  
9   study for HDL-CEC.

10   **RESULTS:** As estimated by NMR-spectroscopy, a 1-SD higher HDL-CEC was associated with  
11   a lower risk of incident CHD (hazards ratio 0.86; 95%CI 0.79-0.93, adjusted for traditional risk  
12   factors and HDL-C). These findings are consistent with published associations based on *in vitro*  
13   cell assays.

14   **CONCLUSIONS:** These corroborative large-scale findings provide further support for a  
15   potential protective role of HDL-CEC in CHD, and substantiate this new method and its future  
16   applications.

## INTRODUCTION

Circulating high-density lipoprotein (HDL) particles mediate reverse cholesterol transport by carrying excess cholesterol from the periphery, such as the arterial wall, to the liver for excretion into the bile. HDL cholesterol (HDL-C) is an established epidemiological risk factor for cardiometabolic conditions (1). However, the role of HDL-C remains unclear since most HDL-C increasing therapies have, on the whole, failed to prevent cardiovascular events (2), and Mendelian randomization studies have given consistent evidence that HDL-C is not causal in the development of cardiovascular disease (3). Even though the recent REVEAL trial (4) of the cholesteryl ester transfer protein inhibitor anacetrapib resulted in a lower risk of major coronary events, rather than providing evidence for a causal role of HDL-C, these findings were entirely proportional to the reduction in apolipoprotein B-containing lipoproteins (5,6).

Therefore, the totality of evidence does not support a causal role for HDL-C in CHD. This has shifted the focus of HDL research from circulating HDL-C concentrations to other aspects of HDL, such as the functional attributes of HDL particles (7,8). Cholesterol efflux capacity of HDL (HDL-CEC), which quantifies the ability of HDL particles to extract cholesterol from lipid-laden cells, has emerged as the most widely used metric for HDL function. HDL-CEC reflects the combined action of various HDL particles via multiple cellular pathways (9): intracellular cholesterol is extracted by HDL via adenosine triphosphate (ATP)-binding cassette (ABC) transporters (ABCA1 and ABCG1), scavenger receptor B1 (SR-B1) and simply by passive diffusion (9). There are multiple cellular efflux assays available either to target specific pathways or their combination (7,10,11). The most common assay to analyze HDL-CEC uses cyclic adenosine monophosphate (cAMP)-treated J774 murine macrophages with radiolabelled cholesterol (10,12-14). HDL-CEC measured in cAMP-treated J774 cells incorporates all the

abovementioned pathways (12). A fluorescence-labelled cholesterol method has also been used (11,14). Despite variation in the relative contributions from the different efflux pathways, the correlation between HDL-CEC quantified by these two assays is quite high (11,15).

In recent years, several studies have investigated the association of HDL-CEC with cardiovascular risk in individuals, with quantification of HDL-CEC mainly by cAMP-treated J774 cells (10,11,14,16). These studies were recently summarized in a meta-analysis that strengthened the evidence that HDL-CEC is inversely associated with cardiovascular risk, with the association being independent of HDL-C concentrations (14). However, results from individual studies were inconsistent (14) and large-scale evidence on HDL-CEC and cardiovascular outcomes is currently limited to two studies, one by Rohatgi et al (11) and the other by Saleheen et al (10). Both of these studies identified inverse associations between HDL-CEC and cardiovascular events independent of established cardiovascular risk factors, including HDL-C and/or apolipoprotein A1 (apoA1). Interestingly, a recent study suggested HDL-CEC to be heritable, independently of HDL-C (17). In epidemiological studies, HDL-CEC is associated moderately with HDL-related parameters, such as HDL-C (10,11,17), HDL size (11,17) and total HDL particle concentrations (HDL-P) (11,17), but weakly with clinical variables (such as BMI and blood pressure) (10,11). Large-scale characterization of the associations of HDL-CEC with multiple cardiometabolic risk factors, as well as HDL subclasses, is currently lacking. The relative paucity of large-scale epidemiology is most likely due to the complexity and cost of cellular HDL-CEC assays. Novel approaches are needed to facilitate such measurements and enable large-scale investigations of the epidemiological role, genetic architecture, and potential causality of HDL-CEC.

To this end, we have developed a high-throughput cost-effective alternative approach to the estimation of HDL-CEC through serum nuclear magnetic resonance (NMR) spectroscopy. Recent advancements in experimentation and automated molecular quantifications have made applications of quantitative NMR in epidemiology and genetics increasingly common in recent years (18,19). These advances have taken NMR-based approaches into large-scale research beyond their well-known role in detailed quantification of lipoprotein subclasses, particles and lipids (18-20). We show here that it is possible to estimate HDL-CEC from serum NMR spectra and that quantification recapitulates the characteristics of *in vitro* HDL-CEC in cAMP-treated J774 cells. This report presents the new high-throughput methodology and confirmatory results regarding the associations of HDL-CEC and incident CHD in a large-scale prospective epidemiological study.

## MATERIALS AND METHODS

An overview of the study is described in online **Supplemental Fig. 1**.

### Training data

Random blood samples were collected during 2016 from the Finnish Red Cross blood service in accordance with the ethical guidelines required by the Helsinki Declaration. Serum was obtained by centrifugation at 1500 x g for 15min at ambient temperature and stored at -80°C. HDL-CEC was measured using cAMP-treated J774 cells within a year of sample collection (Details of *in vitro* HDL-CEC measurement are given in online **Supplemental methods** and **Supplemental Table 1**). The same serum samples were also analyzed by proton NMR spectroscopy within the same time frame. The complete training data set obtained comprised 199 individuals with the serum NMR spectra and the corresponding cellular *in vitro* HDL-CEC estimates. Bayesian



90 regression modelling was applied to link the NMR spectra to the HDL-CEC estimates (21); the  
91 correspondence between the NMR-based and the cellular *in vitro* HDL-CEC estimates are shown  
92 in **Supplemental Fig. 2**. Characteristics of the individuals in the training data set are given in  
93 **Supplemental Table 2**.

#### 95 **NMR spectroscopy and sample preparation**

96 A high-throughput NMR spectroscopy platform with an optimized measurement protocol was  
97 used to provide quantitative information on the multiple molecular constituents of serum. The  
98 experimental details have been previously published (22). The NMR spectroscopic details are  
99 identical to the main quantitative NMR metabolomics method (18, 19) and the overall spectral  
100 characteristics have been detailed and discussed previously (18, 22). Lipoprotein quantification  
101 and HDL-CEC modeling methods are described in the online **Supplemental methods**.

#### 103 **Epidemiological study population and statistical analyses**

104 The FINRISK97 survey was carried out to monitor the health of the Finnish population among  
105 persons aged 25-74 at recruitment (23). The study was conducted in 5 study areas across Finland,  
106 recruiting a total of 8444 persons. The Ethics Committee of the National Public Health Institute,  
107 Helsinki, Finland has approved the study in accordance with Declaration of Helsinki and written  
108 informed consent has been obtained from all participants. Serum samples were collected in 1997  
109 in the semi-fasting state (median fasting time 5h; interquartile range: 4-6 hour) and stored at -70  
110 degrees C. The NMR analyses took place in 2012. Statistical analyses are described in  
111 **Supplemental methods**.

## RESULTS

### HDL-CEC estimation via serum NMR spectroscopy

Using a Bayesian linear regression model, we established a quantitative relationship between the NMR spectral regions of lipoprotein resonances and *in vitro* measured HDL-CEC. We found a good correspondence ( $R^2$  of 0.83) (**Supplemental Fig. 2**) and low mean bias (see Bland-Altman plot in **Supplemental Fig. 3**) between NMR-based HDL-CEC and *in vitro* HDL-CEC.

### Association of HDL-CEC with incident CHD and CVD events

Several studies have found inverse associations between HDL-CEC and cardiovascular outcomes, although results are heterogeneous. We were interested to see whether the analysis using NMR-quantified HDL-CEC would replicate findings by prior studies based on *in vitro* HDL-CEC assays. We analyzed the association of NMR-based HDL-CEC with incident cardiovascular events in a large-scale population-based FINRISK97 cohort (7,290 individuals with 574 incident CHD events and 7,231 individuals with 789 incident CVD events during 15 years of follow-up, online **Supplemental Methods**). The Kaplan-Meier curves in **Fig. 1** illustrate the association of NMR-based HDL-CEC quartiles with risk of incident CHD events during follow-up for the individuals in the FINRISK97 cohort. The HR between the top and bottom quartile of the NMR-based HDL-CEC values was 0.63 (95% CI 0.50-0.80) (**Fig. 1**). The event curves show a dose-response at the median threshold, but the association is non-linear through the quartiles, as reflected also by the cubic spline of the continuous hazard ratio across HDL-CEC values (**Supplemental Fig. 4**). Detailed analyses with multiple adjustments are presented in **Supplemental Table 3**. A similar association was obtained for the association of HDL-CEC with CVD (**Supplemental Fig. 5**). HDL-CEC remained associated with CHD and CVD events even after adjustment for traditional risk factors and all other HDL-related measures, including HDL-

C, apoA1 and total HDL-P; top vs. bottom quartile HR for CHD 0.74 (95% CI 0.57-0.95) (**Fig. 1**) and HR for CVD 0.79 (95% CI 0.64-0.98) (**Supplemental Fig. 5**). The trend to an association for hard atherosclerotic cardiovascular disease, together with MI and stroke separately, were consistent with the association for total CHD (**Supplemental Table 4**). We also analyzed the association of HDL-CEC for incident CHD events across various clinical subgroups (**Supplemental Fig. 6**) and detected no subgroup interactions.

Previously Rohatgi et al (11) examined myocardial infarction (MI) and cardiovascular disease (CVD) in 2,416 individuals over a median follow-up of 9.4 years in a population-cohort with 30 and 172 endpoints, respectively, and Saleheen et al (10) used a prospective nested case-control study with 1,745 patients with incident coronary heart disease (CHD) and 1,749 control participants. We chose these largest HDL-CEC endpoint studies as a reference. **Supplemental Table 5** presents comparisons for the associations of the *in vitro* measured HDL-CEC in cAMP-treated J774 cells with MI and CVD (Rohatgi et al (11)) and CHD (Saleheen et al (10)) outcomes with the current results for the NMR-based HDL-CEC estimates and CHD in the FINRISK97 cohort. All the main associations based on the *in vitro* estimates, including those with various adjustments, were replicated with the NMR-based estimates (see online **Supplemental Methods** for Statistical analyses). Consistent with the findings by Rohatgi et al (11) and Saleheen et al (10) our independent large-scale study also supports the inverse association of HDL-CEC for cardiovascular outcomes. An additional consistent feature in the prior studies together and in our data is that the associations of HDL-CEC with vascular outcomes are robust to multiple adjustments, including HDL-C, apoA1 and total HDL particle concentration (HDL-P).

Rohatgi et al (11) also analyzed the improvement in risk prediction after adding HDL-CEC with

traditional risk factors in prediction models. This resulted in small improvements in all the risk-prediction indexes for the primary end point (consisting of atherosclerotic CVD), including changes in the *c*-statistic from 0.827 to 0.841 ( $P = 0.02$ ), the integrated discrimination improvement index (IDI) of 0.02 ( $P < 0.001$ ), and the net reclassification index (NRI) of 0.37 (95%CI, 0.18, 0.56). In our analyses with well-calibrated models (**Supplemental Fig. 7**), addition of HDL-CEC to traditional risk factors and HDL-C was also associated with small improvements in CHD prediction with improvements in the *c*-statistic (from 0.841 (95%CI, 0.829, 0.854) to 0.843 (95%CI, 0.830, 0.856);  $P = 0.02$  by Student *t*-test for dependent samples:  $t_{\text{stat}} = 1.20$ ,  $df = 7290$ , one-sided), IDI of 0.005 (95%CI, 0.001, 0.015), and continuous NRI of 0.21 (95%CI, 0.05, 0.28). Similar improvements were observed for CVD prediction with *c*-statistics (from 0.837 (95%CI, 0.825, 0.848) to 0.838 (95%CI, 0.826, 0.849);  $P = 0.01$  by Student *t*-test for dependent samples:  $t_{\text{stat}} = 2.29$ ,  $df = 7230$ , one-sided), IDI of 0.004 (95%CI, 0.001, 0.010), and continuous NRI of 0.18 (95%CI, 0.04, 0.26). Hosmer-Lemeshow statistics for model calibration with CVD were  $X^2 = 0.021$ ,  $P = 1$ ,  $df = 8$  and  $X^2 = 0.020$ ,  $P = 1$ ,  $df = 8$  for models with and without HDL-CEC, respectively.

We also wanted to compare how the associations for our FINRISK97 cohort using the NMR-based HDL-CEC estimates compared with those from previous studies, as reported in a recent meta-analysis (14). **Fig. 2** presents the association of HDL-CEC with cardiovascular risk in this study with comparable data from the meta-analysis (14). The results in the FINRISK97 study were remarkably similar to the meta-analysis summary estimates. For example, the RR estimate for the highest vs lowest quartile of HDL-CEC was 0.69 (95%CI: 0.54, 0.87) (**Fig. 1**) in the FINRISK97 cohort, consistent with the meta-analyzed RR estimate of 0.58 (0.39, 0.86) (14). Similarly, the per 1-SD higher HDL-CEC RR estimate of 0.86 (0.79, 0.93) (**Supplemental Table**

3) in the FINRISK97 cohort was nearly identical to the summary meta-analysis RR of 0.87 (0.73, 1.04) (14). For both of these, there was no evidence of heterogeneity between the current HDL-CEC estimates and the pooled estimates from the meta-analysis ( $P$  value 0.46 and 0.91 for highest vs lowest and per-1-SD higher, respectively).

### **Correlations of HDL-CEC with various anthropometric, lipoprotein and clinical variables**

The cross-sectional correlations of NMR-based HDL-CEC estimates with various traits in the FINRISK97 cohort are presented in **Fig. 3** (exact correlation coefficients are given in **Supplemental Table 6**) together with the corresponding values from Rohatgi et al (11) and Saleheen et al (10). In addition, **Supplemental Table 7** describes the various traits according to HDL-CEC quartiles. Analogous to the previous studies, we identified positive correlations of HDL-CEC with, for example, HDL-C, HDL particle size, age, blood pressure, alcohol consumption and female sex. Negative correlations of HDL-CEC were identified for apolipoprotein B, triglycerides, and measures of adiposity. The magnitudes of these correlations were weak, which corresponds with findings reported by Saleheen et al (10). Unlike Saleheen, we did not find negative association between diabetes and HDL-CEC. The correlations of HDL-CEC with HDL-C and HDL particle size were even weaker in Rohatgi et al (11). While HDL-CEC was positively correlated with HDL-C and other HDL-related measures, the highest correlation of HDL-CEC with HDL-C across these 3 studies was 0.4 (**Supplemental Table 6**), supporting the view that HDL-CEC and HDL-C contain largely independent information on HDL metabolism.

### **Association of HDL-CEC with HDL subclasses**

Serum NMR spectroscopy enables the extensive quantification of lipoprotein subclasses, including particle concentrations for four HDL subclasses (18,19). These data were not available

in prior studies including Rohatgi et al (11) or Saleheen et al (10). **Figure 4** shows correlations between the particle concentrations of four HDL subclasses (extra large, large, medium and small) and the HDL-CEC, HDL-C, apoA1 and total HDL-P in FINRISK97 (NMR HDL-CEC) and training data (*in vitro* HDL-CEC). Corresponding correlations stratified by sex are presented in **Supplemental Fig. 8**. HDL-CEC correlated strongly with larger HDL subspecies and the associations between HDL subclass particle concentrations, as well as with other key HDL-related measures, were coherent between the independent data sets. This consistency of association is reassuring since HDL-CEC has been reported to have quite variable associations with HDL-C in different cohorts: the correlation between HDL-CEC and HDL-C was 0.4 in the EPIC-Norfolk study (10), 0.62 in the GRAPHIC cohort (17) and 0.39 in the JUPITER trial data (13). More detailed association analysis between the NMR-based HDL-CEC estimates and HDL subclass lipids are shown in **Supplemental Fig. 9**. The distribution of HDL-CEC values in the FINRISK97 cohort is shown in **Supplemental Fig. 10** and Spearman correlations between the NMR spectral profile and various key HDL-related measures in the training data set are illustrated in **Supplemental Fig. 11**.

## DISCUSSION

We developed a high-throughput method to estimate HDL-CEC directly from serum samples using NMR spectroscopy. The method is based on HDL-CEC estimates from *in vitro* measurements in cAMP-treated J774 cells, the most common technique to analyze HDL-CEC in cardiometabolic research. The NMR-based HDL-CEC estimates appear to capture the same key aspects as the *in vitro* HDL-CEC with respect to associations with various anthropometric, lipoprotein and clinical variables as well as, importantly, with cardiovascular outcomes. We applied the new NMR-based method to estimate HDL-CEC in a large-scale epidemiological study, the FINRISK97 cohort of ~7,200 individuals, among whom 574 CHD and 789 CVD incident events occurred during 15 years of follow-up. This is currently the largest epidemiological study of HDL-CEC, the results of which also show independent inverse associations of HDL-CEC with risk of CHD and CVD lending support to a potential atheroprotective role of HDL function.

The associations of NMR-based HDL-CEC with risk of cardiovascular outcomes were in keeping with summary estimates of a recent meta-analysis investigating the associations of cellular *in vitro* HDL-CEC estimates with cardiovascular outcomes (14). A key finding with the NMR-based HDL-CEC, corroborating the earlier findings with the cellular *in vitro* HDL-CEC in the existing largest outcome studies (10,11) was its association with CHD being independent of other HDL-related measures, including HDL-C, apoA1 and HDL-P. Therefore, the NMR-based HDL-CEC estimates recapitulate the characteristics of cellular *in vitro* HDL-CEC estimates and account for independent information on CHD risk, not captured by other HDL-related measures.

Cardiovascular risk prediction is rarely improved by new biomarkers (24). Currently, the ability

of HDL-CEC to improve CVD risk prediction beyond traditional risk factors has been investigated in two studies, both describing moderate increases in NRI: 38% (16) and 22% (11), but relatively small increases in *c*-statistics. Accordingly, we found an increase in NRI of 21% and small increases in the *c*-statistics. A small increase in *c*-statistic is expected, since this metric is insensitive in model comparisons, when good predictors are already present in the reference model (25).

HDL-CEC is a functional measure related to multiple characteristics of HDL particles, and therefore some level of correlation with other HDL-related measures would be expected. In the FINRISK97 cohort, relatively weak correlations were observed, with the largest being between the NMR-based HDL-CEC estimate and mean HDL-particle size (correlation coefficient 0.31). In Saleheen et al (10) the highest correlation was 0.4 between the cellular *in vitro* HDL-CEC estimate and HDL-C. In the GRAPHIC cohort with HDL-CEC data in 1,988 individuals, a correlation of 0.62 between HDL-C and HDL-CEC was found (17) and in the JUPITER trial data the highest correlation was 0.48 between HDL-CEC and apoA1 (13). Together with the independent associations of HDL-CEC with risk of vascular disease, this points towards HDL-CEC containing independent information on HDL metabolism and reverse cholesterol transport.

The present study is the first large-scale study investigating the association of HDL-CEC with HDL subclass measures. The HDL-CEC estimates associated with very large and large, but not with medium and small, HDL subclass particle concentrations. These findings match previous small-scale studies using cAMP-treated J774 cells (26,27) and the predominance of the associations with larger HDL particles is most likely due to these particles having a larger receiving area for the diffusing cholesterol molecules and thereby more effective mediation of



diffusion than smaller particles (9). This is particularly relevant here since diffusion is thought to be the dominating mechanism for the cholesterol efflux in radioactive cholesterol labelled cAMP-treated J774 cells (28) (see **Supplemental methods**). Diffusion is the main efflux mechanism in cholesterol normal (non-loaded) cells (28,29), indicating that it has a role in maintaining cholesterol homeostasis in cells at basal conditions, whereas, macrophages with cholesterol-loaded states, also known as foam cells, have increased expression of ABC-transporters and enhanced cholesterol efflux through these pathways and decreased contribution of aqueous diffusion-driven pathway (28,29). Currently, we do not know, which pathways are most important in relation to CVD risk (30).

The new cost-effective NMR-based method presented here to estimate HDL-CEC directly from serum samples is designed to correspond to the most common assay to analyze HDL-CEC, i.e., using cAMP-treated J774 murine macrophages with radiolabelled cholesterol (10,12-14). This latter methodology was used by Saleheen et al (10), a large-scale outcome study we compared our current results with. The excellent correspondence of our findings (using the NMR-based HDL-CEC estimates) to those by Saleheen et al (10) (using cAMP-treated J774 murine macrophages), serves to corroborate our approach and is in part expected, given that our NMR assay was developed from the similar *in vitro* assay. In contrast, the other large-scale outcome study by Rohatgi et al (11) applied a less common, fluorescence-labelled (BODIPY) cholesterol method in a similar cell model (11,15). The correlation between HDL-CEC estimates from these two cellular *in vitro* assays is modest (11,15) and it is therefore expected that the NMR-based HDL-CEC results in the FINRISK97 cohort would also match with the results by Rohatgi et al (11) at some extent. However, the main difference between radiolabeled and BODIPY-labelled cholesterol efflux assays is that BODIPY-method favours cholesterol efflux through ABCA1-

transporter (15) that mediates efflux to small HDL and lipid-poor pre-beta particles (31,32), whereas radiolabelled cholesterol assay associates with large HDL particles (26,27) due to movement of radiolabelled cholesterol through all pathways present in cAMP-treated J774 cells, diffusion having the main contributing pathway. Thus, NMR-based HDL-CEC is a proxy for radiolabelled cholesterol efflux assay performed in cAMP-treated J774 cells and it may not represent other efflux models. Despite that, our data are also consistent with the pooled estimate of a recent meta-analysis that summarizes the associations of *in vitro* HDL-CEC estimates with cardiovascular outcomes (14), which serves to further corroborate our NMR-quantified HDL-CEC.

Although estimated HDL-CEC values seem to recapitulate the key characteristics of *in vitro* HDL-CEC, applications of this method to diverse populations, i.e., ethnic subgroups, individuals with extreme lipid values or distinct disease states should be interpreted with care.

Multiple recent drug trials have indicated that increasing circulating HDL-C concentrations does not lead to a reduction in cardiovascular disease (5). Mendelian randomization studies also fail to support HDL-C having a causal role in cardiovascular diseases (3). We should therefore remain sceptical about the potential causality of HDL-CEC. Nevertheless, the new cost-effective NMR-based method to estimate HDL-CEC could be advantageous in widening the research of cholesterol efflux to large population-based cohorts and drug trials and to expedite appropriately powered studies in relation to multiple cardiovascular and metabolic outcomes. Large-scale cohorts with HDL-CEC estimates are needed to investigate and replicate the associations of HDL-CEC with clinical outcomes, and more importantly, to study the genetic determinants of cholesterol efflux in order to perform Mendelian randomization analyses for potential causality.

We propose the new NMR-based method as a pragmatic alternative for HDL-CEC estimates from *in vitro* measurements in cAMP-treated J774 cells, particularly in large-scale epidemiology and genetics. This method appears to have great potential to lower the experimental costs related to HDL-CEC measurements and concomitantly speed-up collection of the extensive epidemiological evidence-base necessary to ascertain whether this functional HDL-phenotype is causal for vascular disease and thus, whether it provides an opportunity for translational applications.

**Author contributions**

S.K. and M.A.-K. conceived and designed the study, interpreted the results and wrote the manuscript. S.K. performed the cellular experiments and the statistical analyses and A.J.K. the spectral modelling and bioinformatics. M.K. and M.T. prepared the samples and performed the NMR experiments. M.V.H., P.O. and J.K. interpreted the results and edited the manuscript. M.P. and V.S. provided samples and the phenotype data of FINRISK97. All authors discussed the results and approved the final version of the manuscript. M.A.-K. supervised the study.

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**FIGURE CAPTIONS**

**Figure 1: Kaplan-Meier cumulative incidence and hazard ratios of CHD by quartiles of NMR-based HDL-CEC.** Hazard ratios were calculated by Cox proportional hazard models with the lowest quartile as the reference group.

HDL-C; high-density lipoprotein cholesterol concentration, apoA1; apolipoprotein A1 concentration, HDL-P; total high-density lipoprotein particle concentration (a sum of the individual HDL subclass particle concentrations), TG; triglycerides.

Model 1: Traditional risk factors (age, sex, geographical region, diabetes, mean arterial blood pressure, blood pressure treatment, smoking, log BMI, total cholesterol, log TG, lipid lowering treatment), HDL-C

Model 2: Model 1, apoA1 and HDL-P

†Additional statistics for Log-rank test (chi-squared = 22.2, df = 3, two-sided)

**Figure 2: Associations of HDL-CEC with cardiovascular risk in the FINRISK97 (NMR-based) compared to previous studies (*in vitro* cellular assays).** Previous studies; risk ratios (RR) and their meta-analyzed results were taken from a recent meta-analysis (14) investigating the association of HDL-CEC with cardiovascular outcomes. Highest vs lowest denote the RR for CVD comparing the highest to the lowest HDL-CEC quantiles defined in each study.

RR from the current FINRISK97 cohort was adjusted for age, sex, geographical region, diabetes, mean arterial blood pressure, blood pressure treatment, smoking, log BMI, total cholesterol, log TG, lipid lowering treatment and HDL-C. Highest vs lowest refers to top vs bottom quartile. There was no evidence of heterogeneity between the current study estimate and the pooled estimate from the meta-analysis by Cochran's Q test, two-sided (P value = 0.46, Q = 0.54, df = 1) and P value = 0.91, Q = 0.01, df = 1) for highest vs lowest and per-1-SD higher, respectively.

**Figure 3: Associations of HDL-CEC with clinical and lipid parameters.** In Rohatgi et al (11) data are Spearman correlation coefficients and in Saleheen et al (10) data were analyzed by linear regression adjusted for age and sex. In the FINRISK97 data are Spearman correlation coefficients adjusted for age and sex;  $n = 7,370$  (exact correlation coefficients and details in online **Supplemental Table 5**).

“-“ in the figure denotes that a correlation coefficient was not available.

<sup>a</sup>association for female sex.

\*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.05$ .

**Figure 4: Associations of HDL-CEC and related measures with HDL subclass particle concentrations in the FINRISK97 cohort ( $n = 7,597$ ) and in the training data ( $n = 198$ ).** The FINRISK97 data are NMR-based HDL-CEC estimates and the training data are from *in vitro* HDL-CEC measurements. Data are Spearman correlation coefficients (95% CI) adjusted for age and sex. Filled symbols refer to  $P < 0.007$  and closed symbols to  $P \geq 0.007$ . HDL subclasses were measured by NMR spectroscopy and are defined by particle size as follows: XL-HDL; very large (average particle diameter 14.3 nm), L-HDL; large (12.1 nm), M-HDL; medium (10.9 nm) and S-HDL; small HDL (8.7 nm) (19).  $P$ -value was adjusted for multiple testing by using principal component analysis (online **Supplemental Methods**). HDL-CEC; NMR-based HDL-mediated cholesterol efflux estimate, HDL-C; high-density lipoprotein cholesterol concentration, apoA1; apolipoprotein A1 concentration, HDL-P; total high-density lipoprotein particle concentration (a sum of the individual HDL subclass particle concentrations).

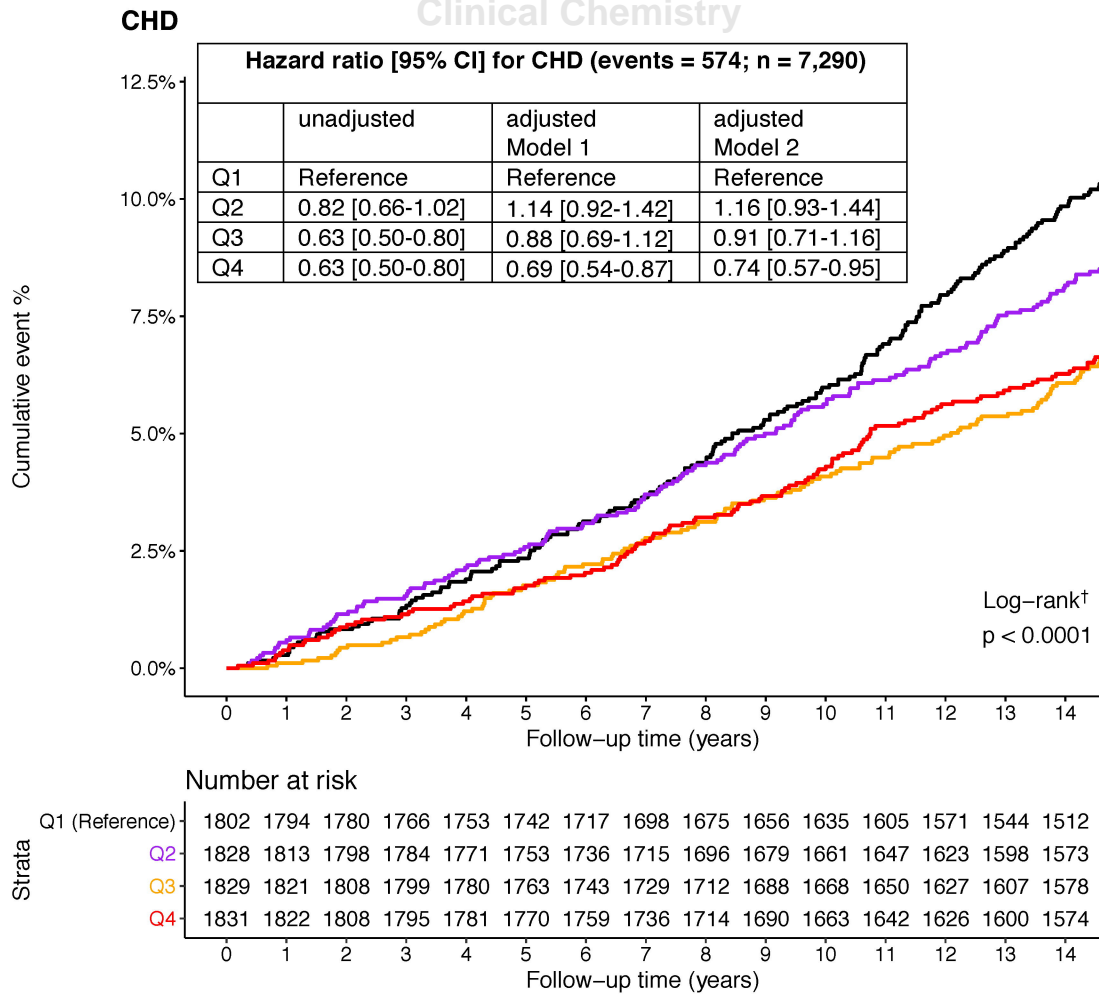


Figure 1

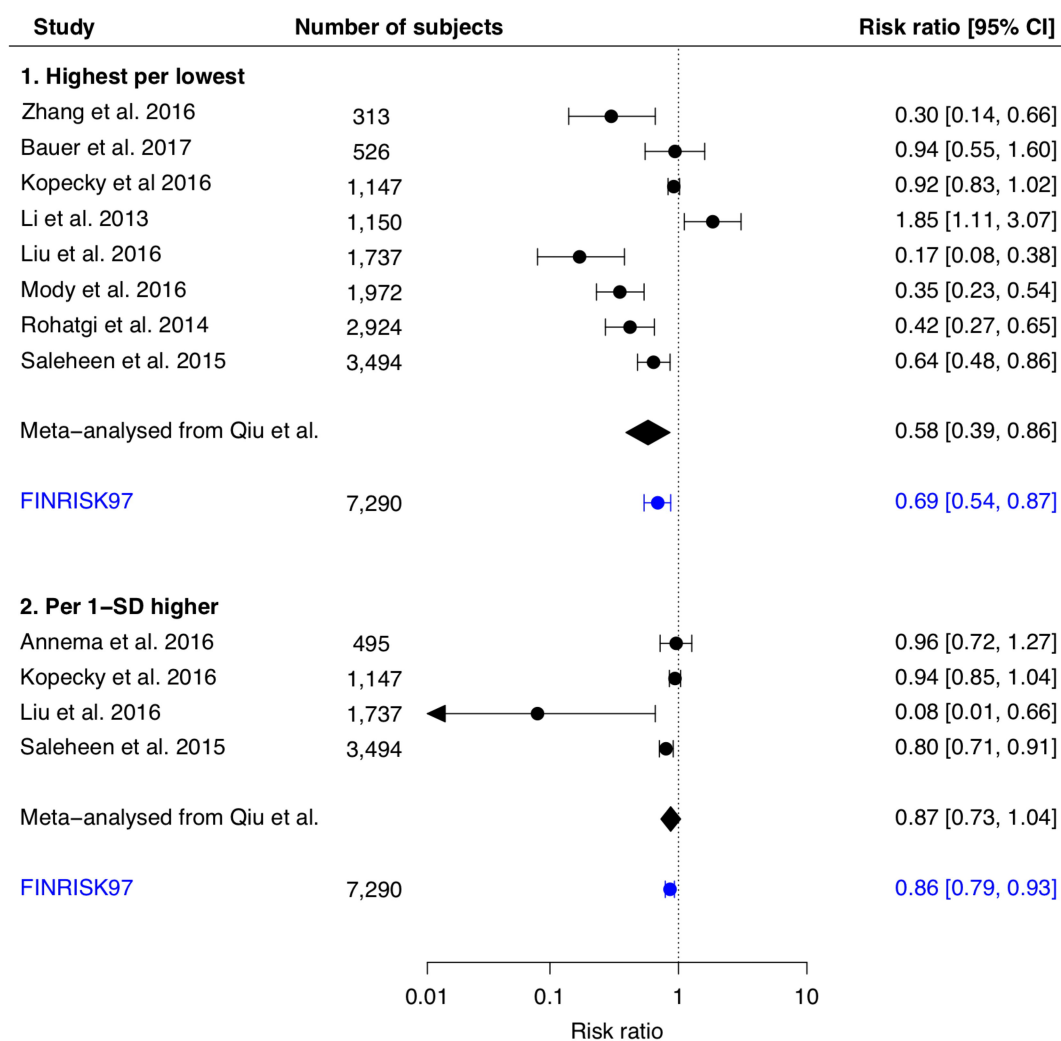


Figure 2.

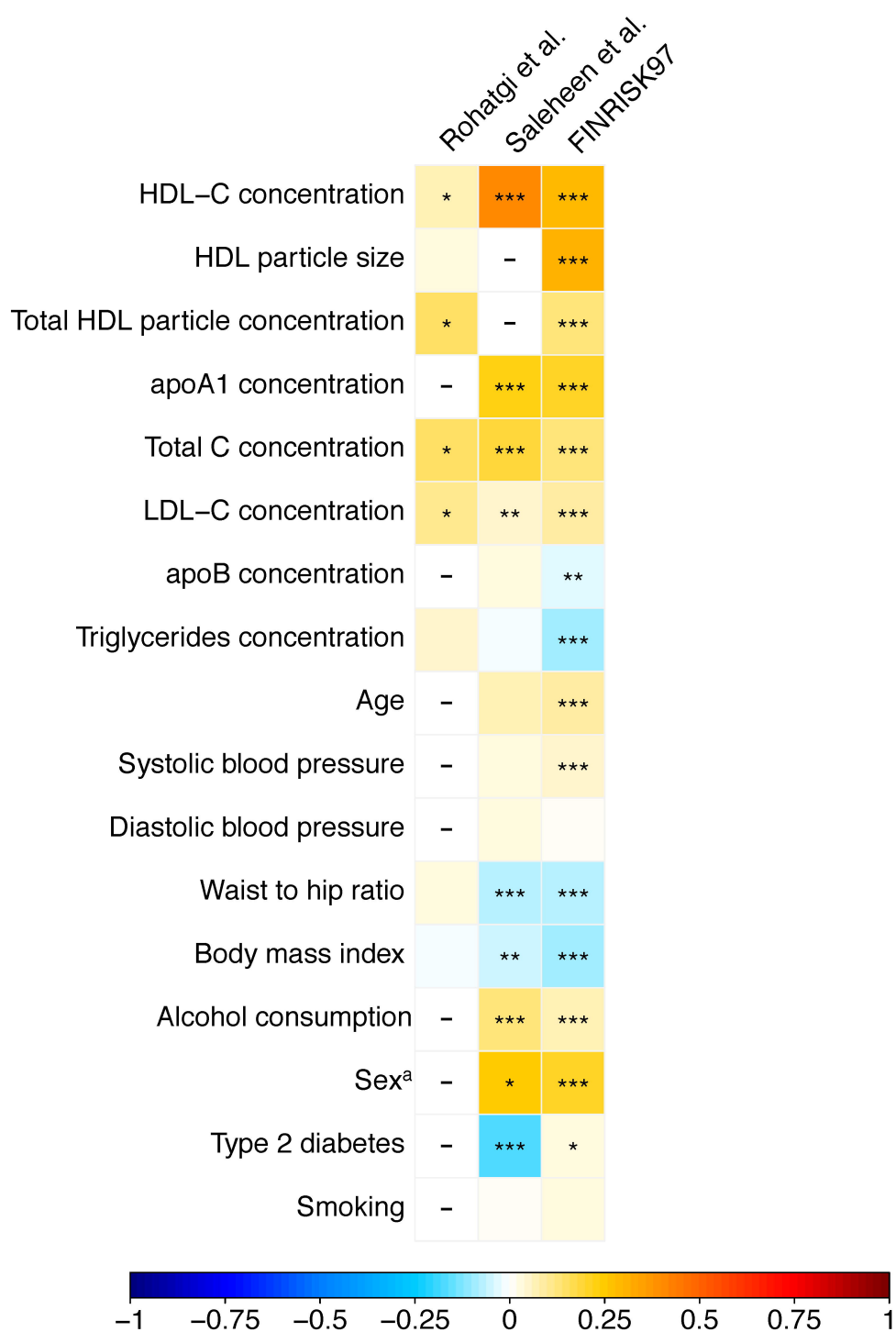


Figure 3.

## Clinical Chemistry

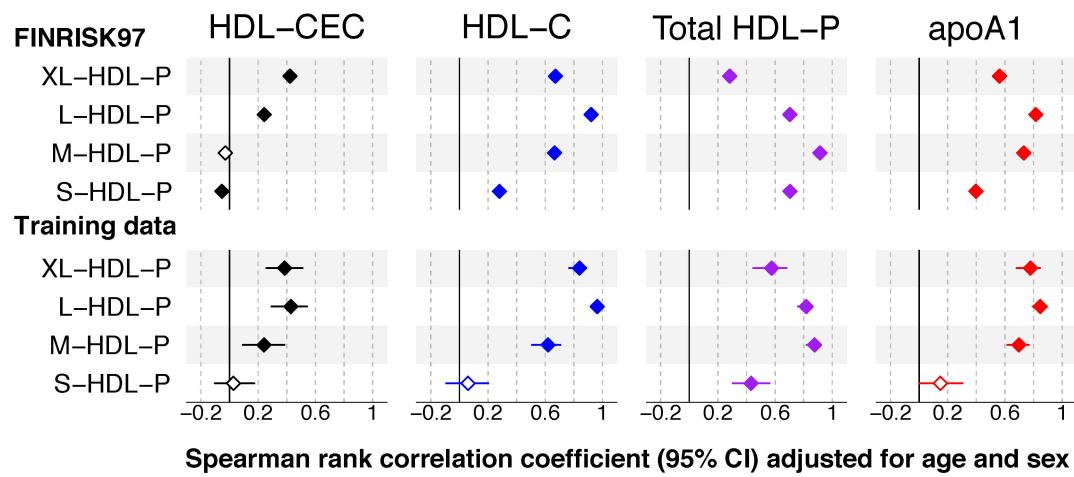


Figure 4, updated